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A preliminary assessment of bacteria in "ranched" ball pythons (*Python regius*), Togo, West Africa

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Abstract

Captive reptiles are routinely identified as reservoirs of pathogenic bacteria and reports of reptile-associated infections relating to some species are well documented (e.g., salmonellosis). Currently, relatively little is known about the epidemiology and bacteria of ball pythons. We carried out a survey of ball python farms in Togo, West Africa to assess the presence of any potentially pathogenic bacterial taxa that have been identified in recent scientific literature relating to this species. The presence of bacteria belonging to the genera *Acinetobacter*, *Bacteroides*, *Citrobacter*, *Enterobacter*, *Lysobacter*, *Proteus*, *Pseudomonas*, *Staphylococcus*, and *Tsukamurella* in oral and cloacal samples taken from five individual ball pythons is of potential concern for horizontal transmission given that pathogenic species belonging to these genera have been previously documented. The presence of bacteria belonging to the genera *Clostridium*, *Escherichia*, *Moraxella*, and *Stenotrophomonas* in the oral and rectal samples taken from five mice used to feed ball

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pythons suggests that they represent a potential reservoir of infection for wild caught ball pythons and their progeny. Furthermore, possible sources of environmental contamination include other captive amphibians, birds, reptiles and mammals, as well as free ranging birds and small mammals. Additional surveillance of ball pythons in the wild and in captivity at python farms in West Africa will shed light on whether or not this type of commercial activity is increasing pathogen exposure and lowering barriers to transmission. Meanwhile, as a precautionary measure, it is recommended that python farms should immediately establish biosecurity and disease surveillance practices to minimize potential horizontal and vertical bacterial transfer.

Keywords
ball python, *Python regius*, reptile, wildlife trade, zoonosis

Introduction

Global demand for reptiles as exotic pets is a relatively recent phenomenon (Mitchell 2009). Their popularity, however, has risen to the extent that they are now thought to represent the second most species-rich vertebrate class (after birds) in the international exotic pet trade (Bush et al. 2014). Reptiles are particularly rife in European and North American markets (Auliya 2003; Jensen et al. 2018), with conservative estimates of c. 0.7 million individuals being kept in the UK and 9.4 million in the USA, respectively (PFMA 2017; APPA 2019).

Global trade in wildlife (whether it legal or illegal) has also been cited as a disease transmission mechanism of growing concern in recent decades (Smith et al. 2009; Can et al. 2019). Specifically, these concerns relate to how pathogens are spread when humans capture wild animals from their natural habitats, transport them by land, sea and air and trade them dead or alive in different parts of a country or the world (e.g., Morens et al. 2004; Karesh et al. 2005; OIE 2017).

Captive reptiles are routinely identified as reservoirs of pathogenic bacteria and reports of reptile-associated infections for some species are well documented, such as salmonellosis (Arena et al. 2012; Bošnjak et al. 2016; Green et al. 2020). Several studies have investigated and highlighted the potential for horizontal and vertical transfer of disease at commercial captive breeding operations [e.g., Green iguanas (*Iguana iguana*) (Mitchell et al. 1999; Mitchell and Shane 2000) and Green sea turtles (*Chelonia mydas*) (Warwick et al. 2013)]. In some scenarios reptile-associated infections can spread to humans who have had direct or indirect contact with pet snakes and feeder rodents (used as reptile food) before their illnesses occurred [e.g., Canada in late 2019; (Government of Canada 2019)].

The ball python (*Python regius*), a species native to western and central Africa, is being exported in relatively large numbers [1,657,814 live individuals since 1978 (Convention on International Trade in Endangered Species of Wild Fauna and Flora [CITES] Trade Database; https://trade.cites.org)]. In fact, it is the single most traded CITES listed species (currently under CITES Appendix II) that is legally exported

alive from Africa (D'Cruze et al. 2020). Much of this international trade can be traced back to a number of python "farms" that are in operation across West Africa, most notably in Benin, Ghana and Togo (Robinson et al. 2015).

Since c. 1996, these python farms have been engaged in "ranching" (UNEP 2019), which refers to *rearing, in a controlled environment, snakes taken as eggs or juveniles from the wild, where they would otherwise have had a low probability of surviving to adulthood* (CITES 2019), and releasing a proportion back into the wild (Ineich 2006). Additionally, gravid females are also collected, and after laying their eggs in captivity are released back into the wild (Ineich 2006).

Recent studies have confirmed that the wild capture of ball pythons (for the export of specimens as "ranched" individuals the CITES source code "R" is used) often involves the removal of snakes from rodent burrows and live transport in sacks filled with other reptiles (D'Cruze et al. 2020). Once at farms, the snakes are reportedly housed separately, but they can also be housed at times in overcrowded enclosures in rooms that are filled with many other reptile species (D'Cruze et al. 2020). Mature ball pythons are typically fed live mice that are sourced from breeders or housed, or even bred, on site at farms.

Despite the international scope, large scale, and national wild release component of ball python "ranching" in Togo, there has been no current research focused on the epidemiology of this commercial trade activity. Therefore, we aimed to carry out an initial review, using amplicon sequence variants (ASVs) methods, to determine the presence of any potentially pathogenic genera of bacteria present in ball pythons and the live mice used as their food. We hope our findings will inform biosecurity surveillance practices to minimize potential horizontal and vertical transfer of zoonotic diseases.

Methods

Literature review

We conducted a systematic review of the scientific literature featured in PubMed, Scopus and Web of Science, from 2009–2019 to identify bacteria that are known to have affected the well-being of ball pythons. The following search terms were used (disease, pathogen, bacteria, bacterial). Each search term was employed with the Boolean operator "AND", with three additional terms (ball python, royal python, *Python regius*).

Laboratory analysis

A total of 20 dry swab samples were taken from five snakes and five mice at a python farm in Togo in September 2019 (Fig. 1, Suppl. material 1). Two swab samples were taken from each animal, one each from oral (both snake and mice), cloacal (snakes only) and rectal (mice only) orifices. Swabs were immediately transferred into 2 ml

screwcap microcentrifuge tubes containing approximately 600 µl of DNA/RNA Shield and stored at -20 °C until transport to the UK.

Prior to DNA extraction, samples (swab and reagent) were transferred into a fresh 2 ml screwcap microcentrifuge bead-beating tube, containing approximately 0.06 g of 0.1 mm glass-silica beads (Thistle Scientific, Glasgow), and vortexed twice for 30 seconds. The swabs were then discarded and the supernatant/liquid portion of the sample transferred to a 1.5 ml tube containing 274 µl polyethylene glycol (6000) and 141 µl 5M sodium chloride and incubated at 5 °C for 15–45 minutes. DNA was extracted using a modified phenol-chloroform method (Rogers et al. 2013) with reduced reagent volumes (200 µl each of molecular biology grade water, phenol, phenol-chloroform and ammonium-acetate: isopropanol compared to the 500 µl used previously). DNA pellets were suspended in 10 µl of molecular biology grade water and stored at -20 °C.

For PCR, 16S rRNA gene amplicons were generated following the Illumina two-step protocol (Illumina 2019) using primers designed by Caporaso et al. (2012) for the first step PCR and Nextera XT Index primers (Illumina, USA) for the second step. PCR conditions are shown in Appendix I. Amplicon clean-up was performed using AMPure XP beads (Beckman Coulter, UK), and normalised using a 96-well Sequal-Prep Normalisation Plate (Thermo Fisher Scientific, USA). Amplicons were sequenced using a 300-cycle MiSeq Reagent Micro Kit v2 (Illumina, USA) with a read length of 2 × 150 bp – on the MiSeq platform.

Sequence quality of the top six samples was visually assessed within R (R Core Team 2019) using the DADA2 R package (Callahan et al. 2016), which was used for taxonomic assignment in combination with the Genome Taxonomy Database (GTDB) (Parks et al. 2018; Chaumeil et al. 2019) using 1 × 150 bp forward read sequences. Raw reads were processed in accordance with the DADA2 Pipeline Tutorial (1.12) (Callahan 2019). In addition to using the standard filtering parameters for trimming, an extra step was added to ensure only amplicons of the expected length were included (i.e., with a minimum length of 148 and a maximum length of 151 bp). The resultant ASV and taxonomy csv files were combined to make a single database, which was further trimmed in Excel (Microsoft, USA). Prior to the assignment of presumptive genus-level classification for the ASVs, NA values for taxonomic levels at family level and above were removed. NA values at the genus level were kept and included as part of the 'other' genus category. Species level assignment was not possible due to the short length of the targeted 16S rRNA region, which may be indistinguishable among species and / or strains (Bulman et al. 2018; Osawa et al. 2015).

Bacterial genera that had been reported in the published scientific literature were identified using the search function within Excel. The assigned identity was confirmed using nucleotide BLAST searches against the 16S rRNA sequences (Bacteria and Archaea) coupled with megablast (highly similar sequences). Sequence read values for each genus were combined to create a stacked bar chart. No cut-off values for reads per sample were applied. The overall relative abundance for some genera were calculated by converting the number of reads for ASVs that have been assigned to a particular genus to a percentage relative to the total number of reads (derived using ASVs assigned genera in addition to the 'other' category).

Results

The literature review identified 29 different species of bacteria across 26 genera that have negatively impacted the health of ball pythons [according to 15 scientific papers published between 2017 and 2019 (Table 1, Fig. 1, Suppl. material 1)]. Sequencing the microbiota of snake and mouse samples collected from the python farm in Togo provided presumptive identification of 13 (50%) of these 26 genera. One of the samples (Mouse Oral 1) did not contain amplifiable DNA and hence was not successfully sequenced. (Table 1, Fig. 1). Searches with BLAST resulted in a query cover range of 94 – 100% and percentage identity range of 89 – 100% (Suppl. material 1).

In terms of overall abundance, 85% of ASVs were assigned to genera of bacteria that were not identified as being of zoonotic concern by the literature review (Table 1, Fig. 1, Suppl. material 1). However, all but one of the samples (95%) contained at least one assigned genus of potential zoonotic concern (Table 1, Fig. 1). Between zero to six of the literature-identified genera were assigned within each sample (mean of two) (Supp. Mat. I). The relative abundance of the literature-identified genera ranged between 0–35% of isolates per sample (mean of 13%) (Suppl. material 1).

Of the literature-identified genera, *Lysobacter* was the most prevalent among the genera-assigned ASVs, although it was only associated with snake samples. *Lysobacter* assigned ASVs also accounted for just over 10% with regards to the overall relative abundance of ASVs. Furthermore, these ASVs were present within eight out of the 19 samples (Fig. 1, Suppl. material 1). The second most abundant genus was *Bacteroides*, which accounted for 2% of the overall relative abundance of ASVs. *Bacteroides* assigned ASVs were present in each of the four sample types (snake oral, snake cloacal, mouse oral and mouse rectal), although relative abundance was greatest in the mouse rectal sample set (Fig. 1; Suppl. material 1).

Discussion

The purpose of this study was to evaluate the presence of any potentially pathogenic bacterial taxa in ball pythons and the live mice used as their food at a commercial python farm that could impact negatively on the health of these snakes and/or those keeping them. The target facility reportedly releases all previously gravid females, and approximately 20% of their hatchlings, back into the wild and exports the remainder internationally for use as exotic pets (primarily to the USA). Of particular interest was relating the epidemiology of infection to potential vertical and horizontal transmission.

This study reported 13 different genera of bacteria, which include species that are known pathogens of ball pythons. The assignment of ASVs to *Acinetobacter*, *Bacteroides*, *Citrobacter*, *Enterobacter*, *Lysobacter*, *Proteus*, *Pseudomonas*, *Staphylococcus*, and *Tsukamurella* in the oral and cloacal samples taken from ball pythons is of potential concern for vertical and horizontal transmission, given that recent scientific literature reports pathogenic species belonging to these genera (Fig. 1, Table 1).

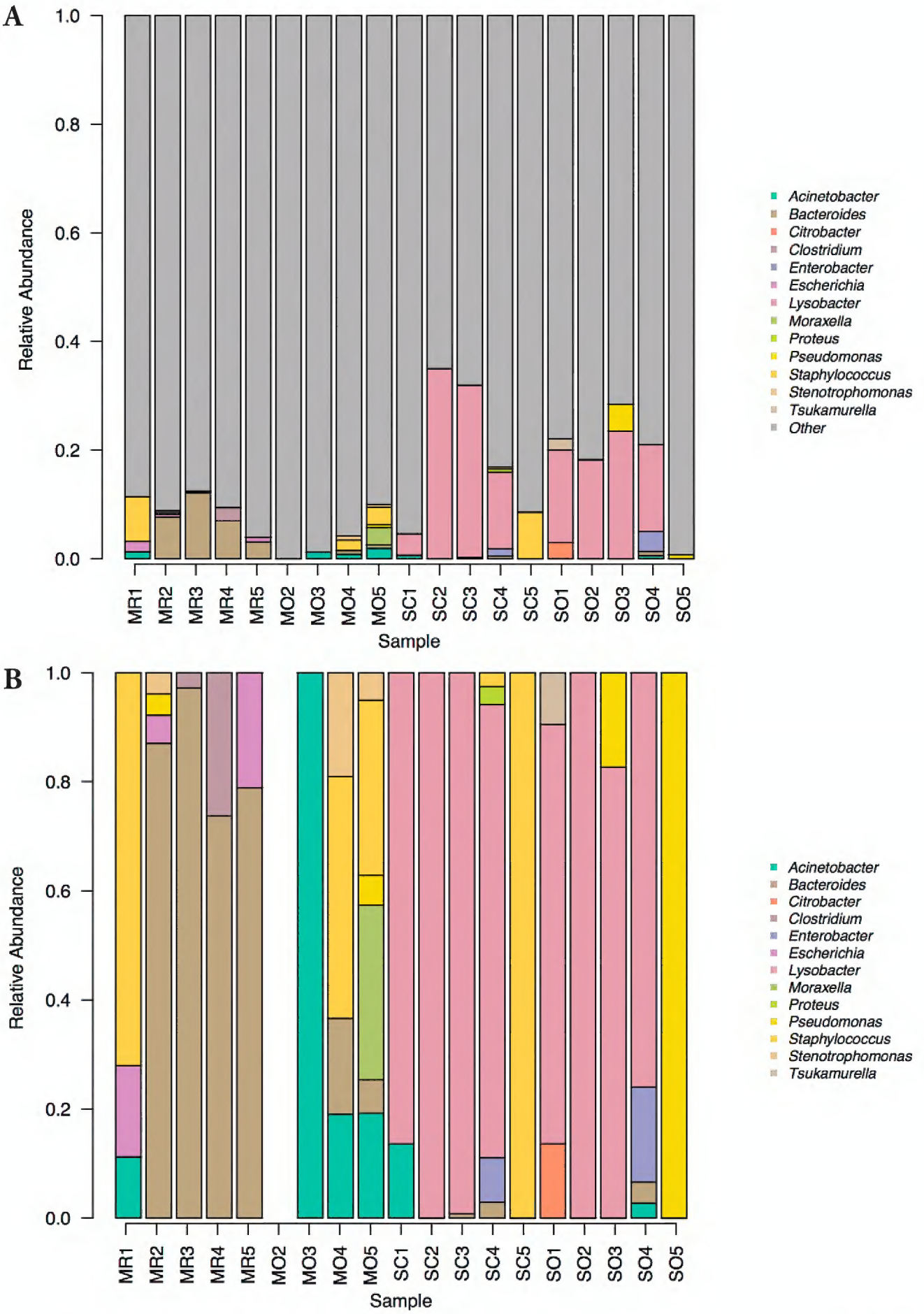

Figure 1.A Relative abundance of bacteria genera identified in samples taken from mice and ball pythons (*Python regius*) at a python farm in Togo. Genera containing potential pathogens of known zoonotic concern to ball pythons (as reported in the scientific literature) are highlighted in colour. The majority of reads (shown in grey) were assigned to "other" least concern groups, which consisted of ASVs that were either assigned to non-target genera or no genus. Samples were from swabs of: MR – mouse rectal; MO – mouse oral, SC – snake cloacal; and SO – snake oral. **B** Relative abundance of bacteria genera of known zoonotic concern to ball pythons (*Python regius*) (as reported in the scientific literature) identified in samples taken from mice and snakes at a python farm in Togo. Samples were from swabs of: MR – mouse rectal; MO – mouse oral, SC – snake cloacal; and SO – snake oral. No bacterial genera of zoonotic concern were identified from sample MO2.

Table 1. List of potentially pathogenic bacteria from ball pythons (*Python regius*) (see Methods).

Genera	Species	References
Acinetobacter	*Acinetobacter calcoaceticus*, *Acinetobacter lwoffii*	Dipineto et al. 2014; Zancoli et al. 2015
Aeromonas	*Aeromonas hydrophila*, *Aeromonas veronii*	Dipineto et al. 2014; Zancoli et al. 2015
Anaplasma	*Anaplasma phagocytophilum*	Nowak et al. 2010
Bacteroides	*Bacteroides* spp.	Dipineto et al. 2014
Bordetella	*Bordetella hinzii*	Schmidt et al. 2013
Chlamydophila	*Chlamydophila* spp.	Hoon-Hanks et al. 2018
Citrobacter	*Citrobacter freundii*	Dipineto et al. 2014; Zancoli et al. 2015; Schmidt et al. 2013
Clostridium	*Clostridium* spp.	Dipineto et al. 2014
Elizabethkingia	*Elizabethkingia meningoseptica*	Schmidt et al. 2013
Enterobacter	*Enterobacter cloacae*	Dipineto et al. 2014; Schmidt et al. 2013
Enterococcus	*Enterococcus pallens*	Zancoli et al. 2015
Escherichia	*Escherichia coli*	Moss et al. 2007; Dipineto et al. 2014; Larsen et al. 2011
Klebsiella	*Klebsiella* spp., *Klebsiella oxytoca*, *Klebsiella pneumoniae*	Bardi et al. 2019; Schmidt et al. 2013; White et al. 2011
Leptospira	*Leptospira grippotyphosa*	Ajayi et al. 2017
Lysobacter	*Lysobacter pythonis*	Busse et al. 2019
Moraxella	*Moraxella osloensis*	Zancoli et al. 2015
Morganella	*Morganella morganii*	Dipineto et al. 2014; White et al. 2011
Mycoplasma	*Mycoplasma* spp.	Hoon-Hanks et al. 2018
Proteus	*Proteus vulgaris*, *Proteus* spp.	Dipineto et al. 2014; Schmidt et al. 2013
Providencia	*Providencia rettgeri*	Myers et al. 2009
Pseudomonas	*Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas japonica*, *Pseudomonas* spp.	Bardi et al. 2019; Zancoli et al. 2015; Sala et al. 2019; Dipineto et al. 2014; Schmidt et al. 2013; White et al. 2011
Salmonella	*Salmonella enterica*, *Salmonella paratyphi* B, *Salmonella* spp., *Salmonella* Muenchen	Moss et al. 2007; Krishnasamy et al. 2018; Dipineto et al. 2014; Schmidt et al. 2013; White et al. 2011
Serratia	*Serratia plymuthica*	Zancoli et al. 2015
Staphylococcus	*Staphylococcus* spp., *Staphylococcus warneri*	Dipineto et al. 2014; Zancoli et al. 2015; White et al. 2011; Schmidt et al. 2013
Stenotrophomonas	*Stenotrophomonas maltophilia*	Zancoli et al. 2015; Schmidt et al. 2013; Klinger et al. 2018
Tsukamurella	*Tsukamurella paurometabola*	Zancoli et al. 2015

The relatively high frequency of ASVs assigned to the genus *Lysobacter* – 80% (n = 8) of oral and cloacal samples from ball pythons – is consistent with a recent report of isolates from the trachea of a ball python suffering from respiratory tract infection [*Lysobacter pythonis* sp. nov. (Busse et al. 2019)]. *Lysobacter* spp. have been described as "ubiquitous inhabitants of soil and water" (Christensen and Cook 1978) making the soil from the dens of wild caught pythons a potential source of infection.

The absence of ASVs assigned to the genera *Citrobacter*, *Enterobacter*, *Lysobacter*, *Proteus*, and *Tsukamurella* in the oral and rectal samples taken from mice used to feed ball pythons suggests that their diet was not a source of infection in this commercial operation, at least on the days of sampling, for these particular genera. Nonetheless, a much larger sample of mice is needed to determine the true bacterial status of the rodents that are typically used to feed "ranched" ball pythons when in captivity.

The presence of ASVs assigned to the genera *Clostridium*, *Escherichia*, *Moraxella*, and *Stenotrophomonas* in the oral and rectal samples taken from mice used to feed ball

pythons suggests that these mice represent a potential reservoir of infection, for these particular genera, that could impact negatively on the health of wild caught ball pythons (i.e., gravid females) and their progeny. Other potential sources of environmental contamination include other captive amphibians, birds, reptiles and mammals that are traded by python farms (cf. Bell et al. 2004), as well as free ranging birds and small mammals (including rodents), which were observed on the farm.

Ball python production systems in West Africa have the potential to encourage disease transmission and the evolution of increased pathogen virulence. Python farms that practice the "ranching" of ball pythons operate at high stocking densities and with poor hygiene measures, where animals are sourced from geographically and ecologically diverse areas with minimal quarantine. These practices can increase pathogen exposure and lower barriers to transmission (Stenglein et al. 2014).

Furthermore, the ball python is the most traded CITES-listed live wild animal currently being exported from Africa, with more than 963,334 snakes exported from Togo alone between 1978 and 2017 (D'Cruze et al. 2020). Commercial breeders in importing countries (predominantly the USA and countries of the EU) also operate at high stocking densities and commonly attend trade shows, where animals from different sources are juxtaposed (Stenglein et al. 2014).

Limitations

The present study was restricted to 20 samples taken from five snakes and five mice at one of the seven python farms currently operating in Togo. Furthermore, it reports only on assigned bacterial genera identified as possessing pathogenic species that are known to have affected ball pythons (as reported by recent scientific literature); thus, this study is not a comprehensive or exhaustive list of genera that may contain zoonotic pathogens. Only 15% of the ASVs in our samples were assigned to genera of concern (as reported in the literature), while other potentially pathogenic genera may be present and could be identified by further analysis.

Species level identification could not be achieved with the samples in this preliminary assessment due to the short length of the targeted 16S rRNA region, which may be indistinguishable among species and/or strains (a low level taxonomic rank used at the intraspecific level) (Bulman et al. 2018; Osawa et al. 2015). Future studies could overcome this limitation and improve taxonomic resolution by using more sensitive techniques, such as quantitative polymerase chain reaction (qPCR) with species-specific primers (Osawa et al. 2015).

Similarly, the present study did not distinguish between pathogenic and non-pathogenic ASVs. This is an important distinction, since the same species of bacteria can act as a harmless commensal, as well as a dangerous pathogen (e.g., *Escherichia coli*) (Proença et al. 2017). To overcome this limitation, future studies should adopt a highly targeted and individual approach per species [e.g., as taken by Delannoy et al. (2017) who detected pathogenic strains of *Escherichia coli* using a qPCR assay that target the

K1 capsule]. Similar analyses should also look to target other pathogen types (e.g., viruses) in "ranched" ball pythons and other wild animal species held in captivity at python farms.

We recognize that the present study represents a preliminary evaluation that should be treated as an initial indicator of both the bacteria present in commercial python farms in West Africa and their potential involvement in zoonotic disease. However, given the international scope, large scale, and national wild release component of the "ranching" process that currently underpins commercial trade of live ball pythons, we believe that these initial findings provide an important insight into the potential for vertical and horizontal bacterial transmission and highlight the need for further research.

Recommendations

Additional surveillance of ball pythons, both in the wild and in captivity at python farms in West Africa, will shed light on whether this type of commercial activity increases pathogen exposure and lowers barriers to transmission. However, in light of other management concerns (Auliya et al. 2020; D'Cruze et al. 2020), and as a precautionary measure, it is strongly advised that farms maintaining reptiles and other wildlife adjust to standard hygiene and quarantine measures, (e.g., biosecurity and disease surveillance practices [cf. Woodford 2000]) to minimize horizontal and vertical transfer.

Biosecurity measures should also be applied to snakes that are being released back into the wild as part of the "ranching" system in Togo. Theoretically, when they are properly released within an area of its indigenous range, this type of wild population "reinforcement" can improve the conservation status of the focal species (IUCN/SSC 2013). However, the IUCN/SSC (2013) recommends effective monitoring as an essential activity, and that reinforcement efforts should include the assessment of disease, welfare conditions, and mortality to maximise positive conservation outcomes.

Biosecurity surveillance practices should extend to importing countries. Such initiatives should also aim to inform those who trade and own ball python of the potential risks associated with zoonotic infection. Providing an appropriate environment and adequate nutrition for ball pythons is also important for maintaining their health. Washing of hands after handling ball pythons is strongly recommended (Centers for Disease Control and Prevention 2018) and they are inappropriate pets for immunocompromised owners and in households with young children (Centers for Disease Control and Prevention 2018).

Conclusion

This survey represents the first investigation into the epidemiology of bacterial genera at a commercial ball python farm in West Africa. This study was developed through the opportunity to collect samples during a broader official scientific review. It is recommended that further research should be carried out at python farms in Benin, Ghana

and Togo. These studies should look to fully assess the species diversity, relative abundance, and pathogenic status of any bacteria (and other types of pathogen such as viruses) present in "ranched" ball pythons and the rodents that are used to feed them.

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Appendix I

PCR conditions for amplicon preparation using the Illumina two-step protocol.

Step 1 Thermocycler conditions			
Stage	**Temperature (°C)**	**Duration**	**Cycles**
Initial Denaturation	98	3 min	1
Denaturation	95	30 s	×25
Annealing	59	30 s	
Extension	72	30 s	
Final Extension	72	5 min	1
Step 2 Thermocycler conditions			
Stage	**Temperature (°C)**	**Duration**	**Cycles**
Initial Denaturation	98	30 s	1
Denaturation	98	10 s	×10
Annealing	62	20 s	
Extension	72	30 s	
Final Extension	72	2 min	1

Supplementary material 1

Genera of bacteria identified in swab samples of mice and ball pythons (*Python regius*)

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Data type: Excel sheet

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